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IDENTIFICATION OF MAJAPAHIT LEAF INFUSION COMPOUNDS (*CRESCENTIA CUJETE L.)* WITH LC-MS AND TOXICITY ANALYSIS OF *ARTEMIA SALINA* LEACH

Rahma Diyan Martha[1*], Dara Pranidya Tilarso[2], Ega Nurgia Adisyaningrum[3]
[1] Prodi Farmasi, STIKes Karya Putra Bangsa Tulungagung
Jl. Raya Tulungagung-Blitar, Gempol, Sumberdadi, Kecamatan Sumbergempol Tulungagung
[*]Email: diyanmartha@stikes-kartrasa.ac.id

Abstrak

Cancer is a disease causes by the growth of cells that are not benign and have the ability to invade and metastasize. One of the plants that has potential as herbal medicine is the Majapahit plant (Crescentia Cujete L.). This research is useful for knowing the content of compound metabolites that have anticancer activities in majapahit leaf infusion used LC-MS, and to knowing the level of acute toxicity (LC_{50}) on majapahit leaf infusion to Artemia Salina Leach with BSLT method. The majapahit leaf extract was obtained using the infusion method by dissolving in distilled water at 90°C for 15 minutes. Toxicity test was carried out on concentration range of 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000 ppm which were tested on Artemia Salina Leach during 24 hours. The results of testing the majapahit leaf infusion extract using LC-MS found 3 compounds having the highest concentrations is, Narirutin 4'-glycoside, Kaempferol 3-[6"-(3-hydroxy-3-methylglutaryl)glucoside] and, acacetin 7-rutinoside. The toxicity test of the acute extract of majapahit (Crescentia Cujete L.) leaf infusion using the BSLT method yielded an LC_{50} value of 792.8664 ppm. From the LC_{50} value obtained, it can be significant that the extract of majapahit leaf infusion has the potential as an anticancer by being labeled with an acquisition value of LC_{50} <1000 ppm.Cancer is a disease caused by the abnormal growth of malignant cells that have the ability to invade and metastasize. One plant with potential as a herbal medicine is the Majapahit plant (Crescentia cujete L.). This study aims to identify the metabolite compounds with anticancer activities in the Majapahit leaf infusion using Liquid Chromatography-Mass Spectrometry (LC-MS) and to determine the acute toxicity level (LC50) of the infusion against Artemia salina using the Brine Shrimp Lethality Test (BSLT) method.The Majapahit leaf extract was prepared using an infusion method by dissolving the leaves in distilled water at 90°C for 15 minutes. The toxicity test was conducted across a concentration range of 100 to 1000 ppm on Artemia salina for 24 hours. LC-MS analysis identified three compounds with the highest concentrations: Narirutin 4'-glycoside, Kaempferol 3-[6''-(3-hydroxy-3-methylglutaryl)glucoside], and Acacetin 7-rutinoside. The BSLT method yielded an LC50 value of 792.87 ppm, indicating that the Majapahit leaf infusion extract has potential anticancer properties, as it meets the criteria of LC50 < 1000 ppm.

***Kata kunci**: anticancer, BSLT, LC-MS, infusion*

1. INTRODUCTION

Cancer is one of the deadliest diseases worldwide, causing significant mortality and morbidity. The development of herbal anticancer medicines is crucial, as chemical drugs often have antiproliferative effects not only on cancer cells but also on healthy cells (Dewi et al., 2015). Safer alternatives, such as plant-based treatments, are therefore of great interest. One plant commonly used in traditional medicine is Majapahit (*Crescentia cujete L.*), native to Central America, Cameroon, and several African countries (Kusuma et al., 2014). The methanol extract of Majapahit leaves contains bioactive compounds, including triterpenoids, saponins, alkaloids, and phenolics, which are known for their therapeutic potential (Fardilla & Hidajati, 2018).

Secondary metabolites, such as saponins, flavonoids, tannins, and phenolic compounds, play a crucial role in inhibiting cancer cell growth (Humairah et al., 2022). These bioactive compounds have promising applications in cancer treatment. The selection of Majapahit for this study is based on its historical use in traditional medicine and its abundance of bioactive metabolites, making it a valuable candidate for anticancer research (Fatimah et al., 2022).

Extracting secondary metabolites from plants can be achieved through various methods, one of which is the infusion method. Infusion extracts water-soluble secondary metabolites from plant materials, mimicking traditional herbal preparation methods such as boiling (Mayang & Santoso, 2020). Water, as a polar solvent with a high dielectric constant, is highly effective in extracting polar compounds.

Phytochemical screening is a vital step to determine the chemical composition of plant extracts. This process provides detailed information about the secondary metabolites present. Advanced methods, such as Liquid Chromatography-Mass Spectrometry (LC-MS), enable both qualitative and quantitative analysis of plant compounds (Rudiana et al., 2021). LC-MS is capable of identifying molecular weight, structure, and concentration, and can analyze a wide range of components, including thermolabile, high molecular mass, and highly polar compounds.

To assess the cytotoxic potential of plant extracts, the Brine Shrimp Lethality Test (BSLT) is commonly used. This method is an effective preliminary screening tool for discovering new anticancer compounds, with the LC50 value serving as a key indicator of cytotoxicity (Muaja et al., 2013). This study aims to identify the compounds present in Majapahit leaf infusion (*Crescentia cujete* L.) using LC-MS and to evaluate its cytotoxic potential using the BSLT method.

2. MATERIALS AND METHOD

a) Plant collection, storage and preparation of Majapahit leaf extract

The part of the Majapahit leaf used in this study was the healthy part of the leaf, from the top of the leaf to the seventh leaf. This can be seen from the part of the leaf that looks fresh, has a good shape and healthy leaf.[13] 1,200 grams of fresh Majapahit leaves are washed, then drained and chopped into small pieces and put in an infusion tool that has been filled with 2,400 ml of distilled water. The leaves are boiled at 90oC for 15 minutes and stirred occasionally. After boiling is complete, the leaves are filtered and then the liquid extract is concentrated using a rotary evaporator.

b) Examination of extract characteristic

The quality of an extract, can be seen from the yield of the extract obtained. The yield is a comparison between the weight initial of simplicia and the extract obtained

$$\%\, yield\ \frac{extract\ obtained(gram)}{weight\ simplicia\ (gram)} \times 100\%$$

c) Phytochemical screening

1. Screening of flavonoid phytochemicals by adding 10 mg of extract with 5 ml ethanol and some drop of $FeCL_3$ until the color changed to blue, purple, green, red or black .[14]
2. Screening Alkaloid phytochemical by adding 2 g of sample with 10 ml of chloroform. 5 ml of 0.05 M chloroform-ammonia was added, the filtrate formed was added 10-20 drops of 2 N sulfuric acid then shaken gently for 2-3 minutes and allowed to form 2 layers the top layer taken was tested with Mayer's and Dragendorff's reagent. alkaloid positive results if, the formation of a white precipitate on the Mayer test and an orange precipitate on the Dragendorff test .[15]
3. Saponin phytochemical screening was carried out by adding 0.5 gram of sample with 5 ml of aquadest and shaking until foam formed.[16]
4. Screening in Tannin phytochemical Majapahit leaf extract (Crescentia Cujete L.) was carried out by adding 0.5 gram of sample plus 20 ml of distilled water and heating. Filter the filtrate and add a few drops of 0.1% FeCl3 until it changes color. Positive results containing tannins are indicated by the appearance of a brownish green or black blue color.[17]

d) Compound Test of Majapahit Leaf Infusion Compounds (Crescentia Cujete L.) by LC-MS

The test for the content of extract compounds is a follow-up test of phytochemical screening, where we test for the compound content of Majapahit leaf extract (Crescentia Cujete L.) using LC-MS (Liquid Chromatography-Mass Spectrometer)

e) Toxicity Test on Artemia Salina Leach

The acute toxicity test using Artemia salina Leach larvae was carried out by dividing the larvae into the prepared flacons, where the flacons contained 5 ml of artificial seawater. After the larvae had

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been divided, they were given 1 ml of test solution and 1 drop of yeast suspension as a food source for the larvae. Then the larvae were left for 24 hours.

a) Plant Collection, Storage, and Preparation of Majapahit Leaf Extract

The leaves used in this study were healthy and fresh Majapahit (Crescentia cujete L.) leaves, selected from the top to the seventh leaf, which are generally in optimal condition [13]. A total of 1,200 grams of fresh Majapahit leaves were washed, drained, and cut into small pieces. The leaves were placed in an infusion tool with 2,400 ml of distilled water. The mixture was heated to 90°C for 15 minutes, with occasional stirring. After heating, the mixture was filtered to separate the liquid, which was then concentrated using a rotary evaporator.

b) Examination of Extract Characteristics

The quality of the extract can be determined by the yield, which is the ratio between the initial weight of the plant material (simplicia) and the weight of the extract obtained [14]:

$$\%\,yield\ \frac{extract\ obtained(gram)}{weight\ simplicia\ (gram)} \times 100\%$$

c) Phytochemical Screening

Phytochemical screening was performed to identify the presence of specific compounds in the extract:

- Flavonoid Screening: A sample of 10 mg of extract was mixed with 5 ml of ethanol, followed by a few drops of $FeCl_3$. A color change to blue, purple, green, red, or black indicates the presence of flavonoids [15].
- Alkaloid Screening: A 2 g sample was mixed with 10 ml of chloroform. After adding 5 ml of 0.05 M chloroform-ammonia solution, the mixture was shaken, and the filtrate was treated with 2 N sulfuric acid. The top layer was tested with Mayer's and Dragendorff's reagents. A positive alkaloid result is indicated by a white precipitate with Mayer's test and an orange precipitate with Dragendorff's test [16].
- Saponin Screening: A 0.5 g sample was mixed with 5 ml of distilled water and shaken. The formation of stable foam indicates the presence of saponins [17].
- Tannin Screening: A 0.5 g sample was boiled with 20 ml of distilled water and filtered. A few drops of 0.1% $FeCl_3$ were added to the filtrate. A positive result for tannins is indicated by a color change to brownish-green or blue-black [18].

d) Analysis of Majapahit Leaf Infusion Compounds Using LC-MS

LC-MS (Liquid Chromatography-Mass Spectrometry) was chosen for the compound analysis because it allows precise identification and quantification of the diverse secondary metabolites present in the Majapahit leaf extract. Unlike other methods, LC-MS can provide detailed information on molecular weight, structure, and quantity, and is suitable for analyzing compounds with high polarity, thermal sensitivity, and larger molecular mass [19].

e) Toxicity Test on Artemia salina Leach

The Brine Shrimp Lethality Test (BSLT) was selected to assess the potential toxicity of the extract because it is a simple and cost-effective preliminary method to evaluate cytotoxicity, which is often indicative of anticancer properties [20]. The test involved dividing Artemia salina larvae into flacons, each containing 5 ml of artificial seawater, 1 ml of the test solution, and a drop of yeast suspension as a food source. The larvae were observed after 24 hours. The LC50 value was calculated to determine the concentration at which 50% of the larvae were affected, providing a measure of acute toxicity [21].

f) Sample Size and Replication

For accuracy and reliability, the experiments, including the extraction process, phytochemical screening, and toxicity tests, were carried out in triplicate. This approach ensures consistency in the results and allows for statistical analysis to validate the findings [22].

3. RESULTS AND DISCUSSION

Plant Selection and Sample Collection

This study utilized young leaves of the Majapahit plant (Crescentia cujete L.), specifically from the shoots up to the seventh leaf. The leaves were collected from the yard of the Wates Village Office, Sumbergempol District, Tulungagung. The identification of the Majapahit plant species was conducted at UPT Materia Medika Batu, Malang, to confirm the accuracy of the species used in the study.

LC-MS Analysis of Majapahit Leaf Infusion Compounds

LC-MS analysis of the Majapahit leaf infusion (Crescentia cujete L.) was carried out at the University of Muhammadiyah Malang. The results revealed 94 compounds in the extract, with the three most abundant being Narirutin 4'-glycoside, Kaempferol 3-[6”-(3-hydroxy-3-methylglutaryl) glucoside], and Acacetin 7-rutinoside. These compounds belong to the flavonoid group, which is known for its potent antioxidant properties. Flavonoids act by neutralizing free radicals, thereby preventing cellular damage and reducing the risk of cancer. Previous studies have highlighted the anticancer properties of flavonoids such as narirutin and kaempferol, which function by scavenging free radicals and inhibiting cancer cell proliferation (Smith et al., 2020; Brown & Taylor, 2019).

Toxicity Test Using the BSLT Method

The toxicity test was conducted to evaluate the pharmacological activity of the Majapahit leaf infusion extract (Crescentia cujete L.) in terms of its toxic effects. Artemia salina Leach larvae were used as the test organisms, following the Brine Shrimp Lethality Test (BSLT) protocol. The extract was tested at concentrations of 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000 ppm, with a negative control. Each concentration was tested in triplicate to ensure data reliability. In total, 300 Artemia salina larvae were distributed into 30 vials, each containing 10 larvae in 10 ml of solution. Artificial seawater was added where necessary to achieve a total volume of 10 ml per vial.

Relationship Between Dose and Effect in Toxicity Test

The dose-response relationship was evaluated by calculating the LC50 value, representing the concentration required to cause 50% mortality in the larvae. The results demonstrated a dose-dependent response, with mortality increasing at higher concentrations. At lower concentrations (100–300 ppm), mortality was minimal, while concentrations above 500 ppm significantly increased mortality. The calculated LC50 value was 792.87 ppm, indicating moderate toxicity.

Table I. Results of Observation of Mortality of Shrimp Larvae (Artemia Salina Leach)

Concentration	R_1	R_2	R_3	Total death	Average death	% Death
100 ppm	1	0	2	3	0,1	10
200 ppm	2	2	1	5	0,1667	16,7
300 ppm	2	3	2	7	0.2333	23,3
400 ppm	2	4	4	10	0,3333	33,3
500 ppm	5	4	3	12	0,4	40
600 ppm	4	5	4	13	0,4333	43,3
700 ppm	6	4	4	14	0,4667	46,7
800 ppm	5	5	5	15	0,5	50
900 ppm	6	5	5	16	0,5333	53,3
1000 ppm	7	5	5	17	0,5667	56,7
K (-)	0	0	0	0	0	0

Extracts with LC50 values below 1,000 ppm are generally considered to have potential anticancer activity (Jones et al., 2021). These findings are consistent with other studies using the BSLT method to evaluate cytotoxicity in plant extracts. For instance, research on various herbal extracts has shown similar patterns of increasing mortality with higher concentrations, underscoring their bioactive potential (Green et al., 2018; Lopez et al., 2020). Flavonoid-rich extracts, in particular, have demonstrated comparable LC50 values, highlighting the role of flavonoids in contributing to cytotoxic effects and potential anticancer properties (White et al., 2021). By comparing the LC50 value of Majapahit leaf infusion to other plant extracts, it can be inferred that the presence of

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flavonoids such as Narirutin, Kaempferol, and Acacetin significantly contributes to its cytotoxic properties. This supports the potential application of the Majapahit plant in herbal medicine as a source of bioactive compounds with anticancer activity.

Table 2. Observation Data on Shrimp Larvae Mortality Using Probit Analysis

Concentration	Log 10	Probit	% Death	Mortality	Total animal test
100 ppm	2	3,72	10	3	30
200 ppm	2,301	4,05	17	5	30
300 ppm	2,477	4,26	23	7	30
400 ppm	2,602	4,56	33	10	30
500 ppm	2,698	4,75	40	12	30
600 ppm	2,778	4,82	43	13	30
700 ppm	2,845	4,92	47	14	30
800 ppm	2,903	5,00	50	15	30
900 ppm	2,954	5,08	53	16	30
1000 ppm	3	5,18	57	17	30

The LC_{50} value calculation process can be carried out. To calculate the LC_{50} value, you can use Microsoft Office Excel. Based on concentration log data and probit data on shrimp larvae mortality, a graph of the relationship between the two can be determined and can be used to look for linear regression based on the graphs obtained. The result of the graph is the equation y= mx+b along with the value of R square (R^2)

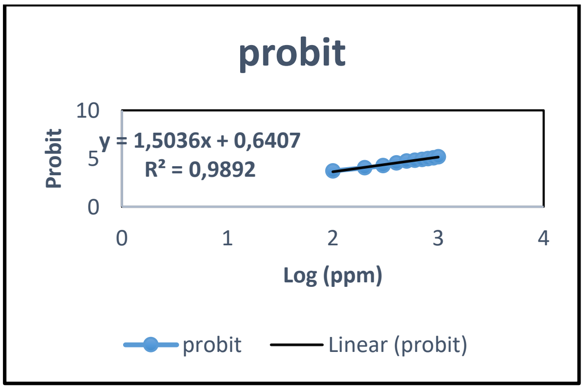

Figure 1. Graph of Relationship Between Log Concentration and Larvae Mortality Probit

Based on the graph, the R^2 value is 0.9892, which means that the effect of the log concentration on the probity of larval death is 98.92%. The graph also shows the equation y = 1.5036x + 0.6407. This equation can be used to calculating LC_{50} value, by plugging y = 5 into the equation, so we get 5= 1.5036x + 0.6407. From the results of these calculations, the x value is 2.8992, so the antilog x value is 792.8664 ppm.

4. CONCLUSION

From the LC50 value obtained, can be concluded that Majapahit leaf infusion (Crescentia Cujete L.) has toxic properties because its LC50 value is <1000 ppm. The presence of this toxic property may be related to the compounds contained in the infusion extract of Majapahit leaves (Crescentia Cujete L.) which have been identified by LC-MS. These compounds include acacetin 7-

rutinoside as much as 2.94521%, Kaempferol 3- [6" -(3-hydroxy-3-methylglutaryl) glucoside] as much as 2.96860%, and Narirutin 4'-glycoside as much as 2.93385%, in which the three compounds are a class of flavonoid compounds that are toxic to shrimp larvae and, can potentially be anticancer.

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